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Dietary Fish Oil Supplementation Reduces Myocardial Infarct Size in a Canine Model of Ischemia and Reperfusion

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Objectives. This study was conducted to determine whether the long-term administration of fish oil attenuates myocardial necrosis in an occlusion-reperfusion model of myocardial ischemia.

Background. Omega-3 fatty acids found in fish oil have various biologic properties that may modify myocardial injury caused by severe ischemia and reperfusion.

Methods. Of 21 dogs fed an identical diet, 10 were given supplemental fish oil containing 0.06 g/kg per day of eicosapentaenoic acid for 6 weeks. Under anesthesia and open chest conditions, the left circumflex coronary artery was occluded for 90 min, followed by 6 h of reperfusion. Regional myocardial blood flow was measured with 15-μm spheres before and during occlusion and during reperfusion. The area at risk and infarct size were measured using standard staining techniques.

Results. In the dogs receiving supplemental fish oil, the platelet cell membrane content of eicosapentaenoic acid increased from 0.9 ± 0.56% to 7.1 ± 4.0% ($p < 0.001$). Infarct size was 29 ± 7% in the control group and 13 ± 3% in the fish oil group ($p < 0.05$). There was no significant difference in the myocardial area at risk or rate-pressure product between the control and fish oil groups. There was no difference in regional myocardial blood flow between the groups at baseline study or during coronary occlusion and reperfusion.

Conclusions. Dietary fish oil supplementation significantly reduced myocardial infarct size in this model. The difference in infarct size did not appear to be related to dissimilarities in regional myocardial blood flow or determinants of oxygen consumption. Further investigation is needed to determine the nature of the protective mechanisms of omega-3 fatty acids on myocardial infarct size.

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Epidemiologic studies (1,2) indicate that death from cardiovascular disease is inversely related to the amount of marine food products consumed. This beneficial effect of seafood has been related to its high content of omega-3 polyunsaturated fatty acids. These fatty acids have significant biologic effects, including alteration in eicosanoid production (3,4) and effects on protein synthesis (5,6), enzyme activity (7) and receptor function (8).

In humans, diets with a high fish oil content modify several risk factors of atherosclerosis. Dietary fish oil supplementation has favorable effects on hyperlipidemia (9) and lowers blood pressure in hypertensive patients (10). Fish oil diets also decrease platelet aggregation (11,12) and modify inflammatory cell function (5,13–15). In animal models, dietary supplementation with fish oil has been shown to retard development of atherosclerosis (16,17). Recent studies (18,19) in humans suggest that the rate of restenosis after coronary angioplasty may be reduced.

In animal models, dietary supplementation with fish oil has been shown to affect myocardial infarct size. Culp et al. (20) demonstrated a reduction in myocardial infarct size after coronary artery occlusion in dogs given supplemental dietary fish oil, and Hock et al. (21) reported a reduced infarct size after coronary occlusion in a rat model. In both of these studies, the investigators used a model of permanent coronary occlusion. However, no information is available on whether dietary fish oil supplementation alters the extent of myocardial necrosis induced by severe ischemia followed by reperfusion.

After coronary thrombotic occlusion, reperfusion of ischemic myocardium occurs frequently, either spontaneously or as a result of therapeutic intervention. Although the net effect of early reperfusion is believed to decrease myocardial damage, several investigators (22) believe that reperfusion may also have deleterious effects. It has been proposed (22) that invasion of inflammatory cells into ischemic myocardium and the formation of oxygen free radicals during reperfusion may promote cell death in otherwise viable myocardium and thus affect final infarct size.

The purpose of the present study was to determine whether dietary omega-3 fatty acid supplementation would reduce myocardial infarct size in a canine model of myocar-

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dial ischemia followed by reperfusion. We supplemented the diet of 10 mongrel dogs with a defined content of eicosapentaenoic acid (20:5 omega-3) and docosahexaenoic acid (22:6 omega-3) for 6 weeks. These animals and 11 control dogs underwent open chest coronary occlusion for 90 min followed by 6 h of reperfusion.

Methods

Feeding protocol. Ten male or female mongrel dogs with an average weight of 15.7 kg received supplemental Max-EPA fish oil for ≥6 weeks. The dosage was 0.06 g/kg per day of eicosapentaenoic acid (20:5 omega-3) and 0.04 g/kg per day of docosahexaenoic acid (22:6 omega-3). The amount of fish oil given was adjusted according to weight every 2 weeks. The oil was mixed with 0.5 oz (14 g) of canned dog food given once a day. Otherwise, the dogs had free access to unsupplemented food. As a control group, 12 dogs of comparable weight were fed the same diet without fish oil supplementation. Five dogs in the control group remained on their diet for 6 weeks before the experiment for better comparison with the fish oil group. The other seven dogs remained on their diet for 1 to 2 weeks before the experiment was performed. There was no significant difference in body weight between the two groups. All animals used in this study were maintained according to a protocol approved by Loyola's Institutional Animal Care and Use Committee.

Sampling of peripheral blood. Baseline blood samples for assessment of platelet aggregation, platelet membrane fatty acid analysis and whole blood viscosity were drawn within 2 days of arrival to our animal care facility. The second set of samples for comparison studies was drawn within 2 days of the experiment. Blood was drawn into plastic syringes through an 18-gauge needle from a superficial foreleg vein.

Platelet aggregation. Nine milliliters of whole blood was drawn into 1 ml of 0.12-mol/liter sodium citrate. The concentration of sodium citrate can affect both platelet yield and platelet aggregation in dogs (23). Despite the lower yield of platelets, we obtained better and more consistent aggregation responses with 0.12-mol/liter sodium citrate. Platelet-rich plasma was prepared by centrifugation at 150 *g* for 15 min at 24°C. Platelets were counted in an automated cell counter (model S plus 4, Coulter). Aggregation studies were performed within 2.5 h of phlebotomy in a platelet aggregation profiler (model-PAP-4, BIO/DATA Corporation). Platelet agonists were collagen (0.8 μg/ml) and adenosine diphosphate (ADP, 12 μmol/liter). These agents give reproducible aggregation responses in dogs (23). The response to ADP was monitored for 3 to 5 min and the response to collagen for 10 to 15 min.

Fatty acid analysis. Blood was collected into sodium citrate and platelet-rich plasma. Butylated hydroxytoluene (0.5%) was added to prevent unsaturated fatty acid autoxidation in all samples. The platelet-rich plasma was washed three times with tris-buffered saline solution (0.05 mol/liter tris-0.15 mol/liter sodium chloride, pH 7.0) and platelets were recovered by centrifugation at 1,800 *g* for 20 min at 4°C. The wet platelet pellet was homogenized in a glass tissue grinder with 2.5 ml of chloroform/methanol 1:1 (volume/volume). The homogenate was transferred to a fritted disk glass funnel and rinsed with 18 ml of chloroform/methanol (1:1), and 10 ml of chloroform was added to adjust the final solvent ratio to 2:1. The extract was dried under nitrogen gas. To remove water-soluble contaminants, 4 ml of chloroform/methanol (2:1) was added to the dried extract, followed by the addition of a 0.8 ml of 0.05 mol/liter potassium chloride. This mixture was mixed by vortex for 10 s, then allowed to phase. The lower chloroform layer was removed and dried and stored under nitrogen gas at −20°C until analyzed. Fatty acids were determined in the platelet lipid extract by gas chromatography. The lipid extracts were suspended in petroleum ether and subjected to transesterification by boron trifluoride methanol. This method is safe for unsaturated fatty acids and complete for methylation of free and esterified fatty acids (24). The methyl esters of the fatty acids were analyzed by gas chromatography on a Varian model 3700 chromatograph (Varian Instruments) fitted with a 4-mm internal diameter column packed with Silar-10c on GCQII, 100–200 mesh (Alltech). The analysis was carried out with a variable temperature program at 180° to 200°C at 1°/min × 20 min. Nitrogen carrier gas was used at 40 ml/min. This program separates arachidonate (20:4) from eicosapentaenoic acid (20:5). Fatty acids are quantified by peak area determination using identical standards for comparison. Pentadecaenoic acid (15:0) was used as an internal standard.

Experimental protocol. A model of open chest anesthetized dogs was used. After anesthesia with alpha-chloralose (75 mg/kg intravenously) and surgical instrumentation as described previously (25), the dogs were stabilized for ≥20 min before the experiment. After heart rate and arterial pressure were recorded, microspheres were injected for initial blood flow measurements. Dogs were injected with lidocaine (2 mg/kg intravenously) just before coronary occlusion to prevent the occurrence of ventricular fibrillation. Lidocaine was administered as needed to either group after reperfusion. The left circumflex coronary artery was then occluded for 90 min by a snare occluder. Although coronary flow was not directly measured in the artery, the entire artery was pulled into the snare and clamped into place for the duration of the occlusion. After 45 min of occlusion, a second injection of microspheres was made. After 90 min of occlusion, the occluder was abruptly released from the circumflex artery. Fifteen minutes after reperfusion, the third injection of microspheres was given. Additional microspheres were injected at 150 and 360 min after reperfusion. Hemodynamic variables and an electrocardiogram were continuously monitored throughout the study and recorded every 30 min. Body temperature was maintained at 37°C, and ventilation was titrated to maintain arterial blood gases within physiologic range. Dogs with ventricular fibrillation that could not be converted into a hemodynamically stable rhythm within 1 min were excluded from the analysis of

Table 1. Platelet Fatty Acid Levels in Fish Oil–Fed and Control Dogs

	Fatty Acid (%)						
	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Arachidonic (20:4)	Eicosapentaenoic (20:5)	All Other Fatty Acids
Control (n = 11)							
Before feeding	15.35 ± 2.2	17.87 ± 4.39	10.7 ± 2.76	14.74 ± 2.78	32.82 ± 3.94	0.8 ± 0.26	7.71 ± 2.46
After feeding	15.51 ± 3.73	21.6 ± 2.75	9.99 ± 1.55	13.33 ± 3.0	31.7 ± 3.24	1.04 ± 0.57	6.82 ± 1.76
Fish oil (n = 10)							
Before feeding	13.23 ± 2.61	20.18 ± 4.31	10.38 ± 1.32	18.02 ± 3.01	29.89 ± 3.22	0.87 ± 0.56	7.43 ± 1.82
After feeding	15.04 ± 5.52	16.93 ± 4.86	12.03 ± 1.9	15.34 ± 3.04	28.08 ± 6.19	7.14 ± 4.0*	5.45 ± 1.9†

*$p < 0.001$, †$p < 0.04$ compared with values in the control group. Data are reported as mean value ± SD.

regional myocardial blood flow and infarct size. Three dogs from the fish oil group and four dogs from control group were excluded from the analysis of hemodynamics, regional myocardial blood flow and myocardial infarct size because these animals had ventricular fibrillation that could not immediately be converted to a hemodynamically stable rhythm. The remaining seven experimental and eight control dogs were used for the analysis of infarct size, myocardial blood flow and hemodynamics.

Regional blood flow and infarct size determination. To determine regional myocardial blood flow, radioactive-labeled microspheres (14.5 ± 0.6; 15 μ) were used (3M Co., New England Nuclear) by methods that have been previously described (26). The equation used to calculate blood flow was: MBF = (Ct/TW) × (RBW/Cb), where MBF = myocardial blood flow in ml/min per g, Ct = tissue radioactivity in counts/min, TW = tissue sample weight in g, RBW = reference blood withdrawal rate in ml/min and Cb = total radioactivity in the reference blood sample. To assess myocardial damage, the heart was excised and placed on a perfusion apparatus. The method for dual perfusion was similar to that previously employed by Lange et al. (27) and modified for use in our laboratory (25,28). The heart was stained with 1% triphenyltetrazolium chloride in phosphate buffer (pH 8.5) solution to reveal the normal tissue (stained in red) and necrotic myocardium (triphenyltetrazolium chloride negative) as previously described (25). The area at risk and the infarct size were calculated as a percent of total area for the surface of each slice. The apical surface area and basal surface area at risk or infarcted was averaged and multiplied by the weight of the myocardial rings to approximate tissue weight at risk or infarct weight, or both. In the apical and basal slices, only the basal and apical surfaces, respectively, were used for the calculation of the weight of infarcted tissue or tissue at risk. With these measurements and the individual weights of the myocardial rings, the following data were derived: area at risk as percent of left ventricular weight, infarct weight as percent of left ventricular weight and infarct size as percent of the area at risk.

Statistical analysis. All data are presented as mean value ± SEM. Analysis between the groups was done by using an unpaired *t* test and, where appropriate, analysis of variance for repeated measures over time.

Results

Platelet lipid analysis. Platelets were analyzed for fatty acid content to demonstrate adequate intake of omega-3 fatty acids and to determine the pattern of fatty acid composition (Table 1). There was no difference in the baseline levels of fatty acids in the fish oil and control dogs. However, after fish oil supplementation, there was a significant increase in the platelet content of eicosapentaenoic acid (20:5 omega-3): 0.9 ± 0.56% before versus 7.1 ± 4.0% after feeding ($p < 0.001$). No significant change in eicosapentaenoic acid was found in the control group platelets. The content of other omega-3 fatty acids, which included the measurement of docosahexaenoic (22:6 omega-3) fatty acid, was not separately determined. In the fish oil group, the total of all other fatty acids showed a relative decrease, indicating a shift in composition from some of the fatty acids in this group to eicosapentaenoic acid. The content of arachidonic acid (20:4 omega-6) did not change significantly in either group (Table 1).

Functional changes in platelets were analyzed by platelet aggregation studies before and after supplementation. The fish oil group showed a significant decrease in the aggregation responses to ADP and collagen. Adenosine diphosphate aggregation response was 60.0 ± 1.7% before feeding compared with 37.5 ± 1.3% after feeding ($p < 0.05$) and collagen aggregation response was 55.9 ± 2.6% compared with 35.2 ± 1.5% ($p < 0.05$). The control group did not show any change in aggregation response.

Infarct size. Myocardial infarct size was significantly decreased in the fish oil group compared with the control group (13 ± 3% vs. 29 ± 7% of the area at risk, $p < 0.05$) (Fig. 1). The amount of myocardium at risk for severe ischemia (expressed as percent of left ventricular mass) from the proximal circumflex coronary artery occlusion was comparable in both the fish oil and control groups (41.2% vs. 39.3%, respectively).

Hemodynamics. There was no difference in heart rate, mean arterial blood pressure or calculated rate-pressure

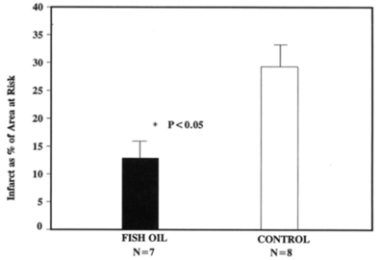
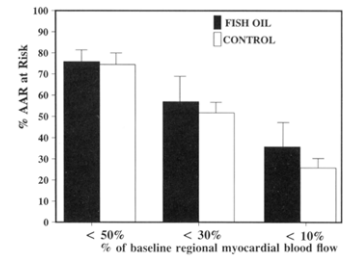


Figure 1. Myocardial infarct size as assessed by triphenyltetrazolium chloride staining in fish oil–fed and control dogs. Results are presented as the proportion (%) of the myocardium at risk found to be infarcted.

product at the time points measured between the fish oil and control groups. There was no difference in the severity of the reperfusion arrhythmias between the two groups.

Blood flow. Regional myocardial blood flow was compared between the two groups at baseline study, during coronary occlusion and three times during reperfusion (at 15, 150 and 360 min). At each time point, blood flow was analyzed as transmural, epicardial, midwall and endocardial. To compare the severity of ischemia during complete coronary occlusion in the two groups, we analyzed the amount of myocardium that had defined reductions in blood flow (Fig. 2). There was no significant difference between the two groups in the quantity of myocardium (determined as a percent of the area at risk) that had ≤50%, ≤30% and ≤10% of baseline myocardial blood flow during coronary occlusion. When endocardial and epicardial blood flow were compared at baseline study (before occlusion) and during occlusion, there was no difference between the fish oil and control groups (Fig. 3). However, at 15 min after reperfusion, endocardial hyperemia was greater in the control group than in the fish oil group ($p < 0.05$), but there was no difference in the epicardial layer (Fig. 3). At 150 and 360 min after reperfusion, there was no difference in epicardial and endocardial blood flow between the two groups (Fig. 3).

Figure 2. Comparison of the severity of ischemia within the myocardium at risk (AAR) between fish oil–fed and control dogs. Radiolabeled microspheres were injected after 45 min of complete coronary occlusion. We analyzed the amount of myocardium (presented as percent of the area at risk) that showed a defined reduction in regional myocardial blood flow (percent of baseline regional myocardial blood flow) during complete coronary occlusion. There was no statistically significant difference between the fish oil and the control groups with respect to the amount of myocardium having <50%, <30% and <10% of baseline myocardial blood flow during coronary occlusion.



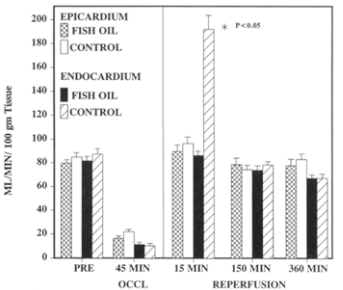


Figure 3. Regional myocardial blood flow (epicardial and endocardial) within the area at risk in fish oil–fed and control dogs. Radiolabeled microspheres were injected and serial analysis was performed at baseline (PRE), at 45 min of coronary occlusion (OCCL) and at 15, 150 and 360 min after reperfusion. The data presented are obtained from analysis of myocardium that had <50% of myocardial blood flow during coronary occlusion. (The amount of tissue with that amount of ischemia was comparable in the two groups [Fig. 2].) The control group demonstrated significantly more endocardial blood flow than did the fish oil group at 15 min after reperfusion.

Discussion

The results of this study demonstrate that dietary supplementation with fish oil reduces myocardial necrosis after 90 min of coronary occlusion followed by 6 h of reperfusion in an open chest canine model. Culp et al. (20) previously demonstrated that dietary fish oil supplementation for 4 to 6 weeks resulted in smaller infarcts after 24 h of coronary artery occlusion in preinstrumented conscious dogs. Hock et al. (21) also reported that after 4 weeks of fish oil supplementation, anesthetized rats had a significant reduction in the loss of creatine kinase after 6 h of permanent coronary ligation.

Effects of omega-3 fatty acids. The mechanisms by which fish oil supplementation mediates protective effects against

myocardial necrosis induced by ischemia, whether or not it is followed by reperfusion, are currently incompletely understood. However, omega-3 fatty acids, which are found in high quantities in marine oils, have a variety of biologic effects that may affect the diverse pathophysiologic processes operating during myocardial ischemia and reperfusion.

Probably the best described effect of omega-3 fatty acids is their modification of eicosanoid metabolism. Incorporation of eicosapentaenoic acid (20:5 omega-3) into cell membrane phospholipids causes competitive inhibition of cyclooxygenase and decreases the conversion of arachidonic acid (20:4 omega-6) to prostaglandins (12,29). Similarly, eicosapentaenoic acid also inhibits the formation of leukotrienes by lipoxygenases (13–15). Simultaneously, eicosapentaenoic acid acts as a substrate for these enzymes and is metabolized to triene prostaglandins and modified leukotrienes, many of which have significantly less biologic activity than their omega-6 derivatives (29–31).

Studies that have focused on the effect of cyclooxygenase inhibitors on infarct size in animal models have failed to show any consistent benefits (32–34), with the exception of ibuprofen (35,36). However specific thromboxane A_2 synthetase inhibitors (37) and receptor blockers (38,39) have been shown to reduce infarct size in dogs, suggesting an important role of thromboxane A_2 in the pathophysiology of myocardial ischemia. Studies (40) utilizing BW 755C, an inhibitor of both cyclooxygenase and lipoxygenase, have also shown reduction in canine infarct size, indicating the significance of lipoxygenase products. Other studies have shown a reduction in infarct size in models of ischemia and reperfusion after the use of a peptidoleukotriene antagonist (41) and specific lipoxygenase inhibitors (42).

Eicosapentaenoic acid affects both cyclooxygenase and lipoxygenase activity and causes a significant decrease in thromboxane A_2 and leukotriene production. Therefore, it can be proposed that at least part of the beneficial effects of fish oil supplementation on infarct size is mediated through its modification of the eicosanoid metabolism.

Myocardial blood flow. Other studies indicate that fish oil supplementation may increase the release of endothelium-derived relaxing factor (43) and this effect would favor coronary vasodilation and increase blood flow as well as inhibit platelet aggregation. However, despite finding antiaggregatory effects on platelets (confirming the biologic effect of the feeding protocol), we found no favorable effects on regional myocardial blood flow during coronary occlusion in the fish oil group compared with the control group. Therefore, the difference in infarct size observed in this study cannot be explained by the difference in ischemic insult between the groups.

We had also proposed that myocardial blood flow after reperfusion might improve by promoting coronary vasodilation and inhibiting platelet and neutrophil aggregation. However, there was no significant difference in regional myocardial blood flow during the reperfusion period except at 15 min of reperfusion, where we found an increased hyperemic response within the endocardium in the control group. The finding of more endocardial hyperemia early after reperfusion in the control group is in contrast to the observation of Hartog et al. (44). In a model of open chest anesthetized pigs, they observed that fish oil supplementation increased the hyperemic response during reperfusion after 15 min of coronary occlusion. These conflicting data may be attributed to differences in experimental design (that is, dissimilar ischemic time) or may be related to variations between animal species. Whether the endocardial hyperemia at 15 min produced additional "reperfusion injury" in the control dogs and therefore relative myocardial protection in the fish oil group can be speculated.

Hemodynamics. In this study, we also found no significant difference between groups in heart rate, blood pressure or rate-pressure product. Thus, the dissimilarity in infarct size between the groups cannot be explained by variation in oxygen demand between the groups. Without a clear difference in ischemic time or oxygen demand to explain the dissimilarity in infarct size, an alternative hypothesis is needed. It is possible that the anti-inflammatory effects of omega-3 fatty acids are important. Inhibition of neutrophil infiltration into severely ischemic myocardium has been shown to reduce myocardial infarct size (45–47). Thus, by inhibiting prostaglandin and leukotriene formation, decreasing platelet-activating factor production (48), reducing cytokine formation (5) and decreasing free oxygen radical formation by neutrophils (49), omega-3 fatty acids may decrease chemotaxis and favorably modify the inflammatory reaction in the myocardium after ischemia with or without reperfusion.

Conclusions. Our study demonstrates that omega-3 fatty acid supplementation can reduce myocardial necrosis in a canine model of ischemic injury and reperfusion. We did not find convincing evidence that the protective effect of omega-3 fatty acids was due to preserved myocardial regional blood flow or alteration in hemodynamic responses to ischemia. Further investigation is needed to determine the protective mechanisms of omega-3 fatty acids on myocardial infarct size.

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